Using nematode communities to test a European scale soil biological monitoring programme for policy development

Stone, D; Costa, D; Daniell, TJ; Mitchell, SM; Griffiths, BS; Topp, CFE

Published in:
Applied Soil Ecology

DOI:
10.1016/j.apsoil.2015.08.017

Print publication: 01/01/2016

Document Version
Peer reviewed version

Using nematode communities to test a European scale soil biological monitoring programme for policy development

Stone, D.[1, 2*], Costa, D.[3,4], Daniell, T.J.[5], Mitchell, S.M.[5], Topp, C.F.E.[6], Griffiths, B.S.[6]

[1]Faculty of Biological Sciences, Leeds University, Leeds, UK, [2]Teagasc, Johnstown Castle Research Centre, Ireland, [3]Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Coimbra, Portugal, [4]IMAR, Department of Life Sciences, University of Coimbra, Coimbra, Portugal, [5]The James Hutton Institute, Invergowrie, Dundee, UK, [6]Crop & Soils Systems Research Group, SRUC, Edinburgh, EH9 3JG, UK,

*Corresponding author (d.g.stone@leeds.ac.uk)

Abstract

There is a current need to identify European biological indicators of biodiversity and ecosystem function that can be used for soil monitoring, in order to aid policy making. Europe, however, is subdivided into different bio-geographical (climate) zones, containing different soils and varying management practices. This work (as part of the EcoFINDERS project) set out to determine the range of variation in nematode community structure as a potential indicator across European bio-geographical zones, taking into account land use and soil characteristics. Nematodes have been suggested as biological indicators for the monitoring of soil quality due to their involvement in the delivery of functions such as carbon sequestration and recycling of nutrients as well as the provision of habitat for biodiversity. Using a molecular (directed-T-RFLP) approach for rapid nematode community structure assessment and a traditional morphological assessment at a

feeding group level, we determined that nematode communities differ between bio-geographical zones and between different land uses within bio-geographical zones. Therefore, at the very large or trans-national level, the presence of any differing bio-geographical zones within the monitored area should be taken into account when sampling and analysing data. Care should be taken when making comparisons across different bio-geographical zones.

Keywords: Nematodes, Nematode communities, large scale sampling campaign, T_RFLP,

1 Introduction

Since the development of the Soil Thematic Strategy (EU, 2002) there has been increasing interest in the links between both soil biodiversity and soil ecosystem service provision, and soil quality (Ritz et al., 2009). Subsequent developments in environmental monitoring and risk assessment are moving toward the use of indicators and endpoints that are related to soil functioning and ecosystem services (Faber et al., 2013). Currently there is no comprehensive indicator of soil biodiversity that can combine all the different aspects of soil complexity in a single formula thus allowing accurate comparisons (Turbé et al., 2010). In response to this problem, it has been suggested that a suite of indicators should be used (Faber et al., 2013, Stone et al., 2015). Soil nematodes are recognised as potentially useful indicators due to their high sensitivity to perturbations and disturbances (Chen et al., 2010). Nematodes are present in all trophic levels of soil food webs making them a good indicator for the functions of carbon sequestration and recycling of nutrients as well as involvement in the function of provision of habitat for biodiversity (Ritz and Trudgill, 1999; Chen et al., 2010, Griffiths et al., 2012).

Nematodes have been used as biological indicators across individual countries for some time (Faber et al., 2013; Stone et al., 2015). However, a greater level of detail regarding the range of biodiversity present across all European bio-geographical zones, land uses and soil types is needed to aid European policy makers in the development of soil policy. In 2001, the OECD identified that to improve the interpretation of biodiversity indicators there was a need for information on their spatial and temporal coverage, including not only species presence, but also changes in species abundance and their distribution (OECD, 2001). This information should be as overall trends rather than absolute values. Specifically, if baselines could be established for the indicator measured, this could help improve the assessment of progress towards current goals and therefore the establishment of future targets. If nematodes are to be used as an indicator for soil biodiversity and ecosystem function across Europe, nematode communities need to be assessed across a range of European soil, land use and climate characteristics. The sensitivity of nematodes as an indicator should be able to reflect the influence of management and climate on long-term changes in soil quality (Breure, 2004).

Molecular methods of identifying soil dwelling nematodes for the purposes of assessing nematode communities are in an exciting period of development. The traditional method of morphological identification to genus or species by microscopic examination of a subset of the extracted community is still used, but there has been a recent increase in the development and use of molecular based approaches as the technology has advanced and become quicker and cheaper to use (Chen et al., 2010, Donn et al., 2012, Porazinska et al., 2012, Yang et al. 2013). To take account of this transition in the use of morphological and molecular methods, both types of analyses were performed on nematodes extracted from the sampled sites of the EcoFINDERS

transect (Stone et al, 2015) to provide a pool of nematode community data that could be compared and used interchangeably.

Our hypothesis was that there were characteristic nematode communities according to bio-geographical zones, land management schemes and soil types. Such information would be relevant to inform the design of future, European scale, biological monitoring schemes.

2 Method

A transect of 81 sites were sampled across European climatic or bio-geographical zones (Figure 1). Due to sampling constraints, one composite sample was collected from each site, with no replication A detailed outline of the sample sites is given in Stone et al. (2015). Each site was sampled following a pre-agreed standard operating procedure (SOP) whereby 20 cores of 5 cm diameter and 5 cm depth were collected at random within a 2 m^2 area chosen as typical for each of the 81 sites (Stone et al., 2015). Cores were transported to a central handling facility at 4 ºC where a single, composite sample for each site was prepared from 12 of the 20 cores. The composite sample was broken up by hand and mixed using the cone and quarter method (Massey *et al*., 2014). From this composite sample, 100 g of fresh soil was subsampled for nematode elutriation. At the same time, a second subsample of 30 g was taken for moisture content determination. Nematode extraction with an Oostenbrink elutriator was performed following an adapted version of ISO 23611-4:2007(E) where the suspension of nematodes and small soil particles were passed through four sieves of decreasing mesh width (mesh width: 180 µm, 120 µm, 95 µm and 45 µm pore size respectively). The catch was then washed from each sieve onto tissue filters mounted on supporting sieves within Baermann funnels of water and left at room temperature for 48 hours. During this time the nematodes separated themselves from the debris

on the filter through active downward movement and were captured in water in 50 ml centrifuge tubes. Nematodes were allowed to settle for 24 hours at 4 ºC and the supernatant then removed by careful pipetting to leave 4 ml of nematode sample.

Extracted nematodes were sub-divided into two samples (A and B) in separate micro-centrifuge tubes. Nematodes were once again allowed to settle for 6 hours at 4°C and the supernatant then removed by careful pipetting to leave 0.5 ml of nematode sample in each tube. Nematodes in sample A were frozen and stored for DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis. Nematodes in sample B were fixed in DESS, following the method of Yoder et al. (2006), for counting and morphological identification to trophic group level.

Genomic DNA was extracted from sample A using a Purelink® Genomic DNA Kit (Invitrogen) according to the manufacturer's protocol for Mammalian Tissue and Mouse/Rat Tail Lysate. DNA was eluted in 50 µl Tris Buffer (10 mM Tris-HCL pH 8.0) and then stored at –20 ºC until used as a PCR template for directed-TRFLP as described by Donn et al. (2012).

DNA (18S rDNA) was selected for amplification using the primers: Nem_SSU_F74 (AARCYGCGWAHRGCTCRKTA) (Donn et al., 2011) and fluorescently labelled FAM-Nem_18S_R (GGGCGGTATCTRATCGCC) (Floyd et al 2005) (Eurofins MWG Operon, Ebersberg, Germany). PCR amplifications of 1.2 µl genomic DNA template were performed in 15 µl final volume reactions containing 1.5 µl of x10 PCR buffer (Bioline, London, UK) with 2 mM $MgCl_2$ (0.6 µl 50 mM $MgCl_2$), 0.3 µl each of 10 mM dNTP mix and BSA, 0.45 µl of each primer (10 pmol/µl) and 0.12 µl of *Taq* polymerase (0.6 units). The volume of template DNA was as used by Wiesel et al. (2015) and yielded robust PCR amplification. All PCRs were performed on a G-STORM Thermal Cycler (Gene Technologies Ltd., Braintree, Essex, UK).

114 The thermal cycling involved one initial denaturation cycle at 94 ºC for 2 minutes, followed by
115 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 51 ºC for 30 s, and extension at 68
116 ºC for 30 seconds. A final elongation step was performed at 68 ºC for 10 minutes. Positive
117 (DNA extracted from mixed nematodes, confirmed by preliminary study) and negative (distilled
118 water) controls were included for each amplification series.
119 The amplified DNA then underwent T-RFLP analysis in a dual enzyme sequential digest. Firstly
120 a PleI enzyme mix, made up of 1 x NEBuffer4 (20 mM Tris-acetate, 10 mM magnesium acetate,
121 50 mM potassium acetate, 1 mM dithiothreitol (pH 7.9)), 100 μg ml^{-1} BSA (supplied with the
122 enzyme) and 2 units PleI per μl (all reagents from New England Biolabs, Hitchin, UK), was
123 added to 10 μl PCR products which were digested at 37°C for 60 min, followed by 65°C for 20
124 min, to denature the enzyme. Digested products were then digested in a BtsCI enzyme mix,
125 made up of 1 x NEBuffer4 (as above), 100 μg ml^{-1} BSA (supplied with the enzyme) and 2 units
126 BtsCI per μl, with incubation at 50°C for a further 1 h. Products were then frozen at -20 ºC to
127 inactivate the BtsCI enzyme and transported to the James Hutton Institute, Dundee, UK in dry
128 ice. Digest products were diluted 1 in 10 and subsequently 1 μl of this dilution was mixed with 9
129 μl Hi-Di™ Formamide and 0.05 μl ROX labelled MapMarker 1000 (BioVentures, Murfreesboro,
130 Tennessee, USA). Fragments were then analysed on an ABI 3730 capillary sequencer (Applied
131 Biosystems, Foster City, CA, USA). Output was processed using a genemapper (Applied
132 Biosystems), followed by calculation of relative abundance and removal of peaks representing
133 less than 1% abundance within any sample as described in Donn et al., (2012).
134 Nematode community data translated from T-RFLP peaks, previously identified as representing
135 nematode taxa isolated from UK soils at the family level (Donn et al., 2012), were assigned to

major trophic groups characterized by feeding habits: bacterial-feeders, fungal-feeders, plant-feeders, omnivores and predators.

For morphological analysis of sample B, the nematode samples were shaken to homogenise the content and a 200 µl subsample was placed in a Doncaster counting plate (Doncaster 1962). The nematodes were counted and identified at the trophic level (bacterial-feeders, fungal-feeders, plant–feeders, omnivores and predators) by observing the head/ mouth structures under an inverted microscope (100x and 200x magnification). The 200 µl subsample contained an average of 480 nematodes (range 40 – 1600), with either all the nematodes or a maximum of 200 being identified as appropriate. Results were converted to the total volume of sample and then soil moisture content values were used to report numbers per gram of dry soil.

Due to the sampling constraints that dictated that each site could only be sampled once with no on-site replication, sites were grouped by biogeographic region, land use, and pre-set ranges of soil texture, pH and organic matter content. Replicates within groups were compared. Multivariate analysis (Principal Component Analysis, PCA, using Genstat version 16) was performed on the nematode community structure as reported from both the T-RFLP and morphological identification. Factor scores from the PCA analysis of both T-RFLP and morphological identification were run through analysis of variance (ANOVA) by soil type, biogeographic region and land use. Tukey multiple comparison tests were carried out on means indicated to be significantly different by ANOVA. A multivariate analysis of variance approach for unbalanced designs (RMULTIVARIATE, Genstat 16) and multivariate linear mixed models (MLMM) were used to compare the effect of the biogeographical zone, land use, soil texture, soil carbon and pH on the nematode communities as reported by both the T-RFLP score and the morphological identification. In both cases, only three-way interactions were included in the

analysis and combination of variables that had no effect on the significance were excluded in a step-wise manner. Results were considered statistically significant at $p < 0.05$. A linear regression model was used to assess the effects of biogeographical zone, land use, soil texture, soil carbon and pH on nematode abundance in the soil. In order to meet the assumptions of the analysis, the square transformation was applied to the data. Results were considered statistically significant at $p < 0.05$.

3 Results

The 81 sites sampled provided a range of bio-geographical zones, land uses and soil types (Table 1) representative of the European situation (Figure 1, and see Stone et al., 2015 for more in-depth analysis).

Nematode community structure differed between bio-geographical zones and between land use within bio-geographical zone (Figure 2 and 3). Variation around means was high, but significant differences between nematode communities were found using both T-RFLP and morphological assessment at a ($P<0.01$). Data from T-RFLP showed that nematode community structure differed between Boreal and all other bio-geographical zones ($P<0.01$), while data from morphological assessment showed that the nematode communities from Boreal, Continental and Mediterranean were separated from all other bio-geographical zones, with the Atlantic and Alpine communities being similar (Figure 2)

Grouping sites into combinations of bio-geographical zone and land use produced tighter clusters (significantly different means at $p<0.05$ from both T-RFLP and morphological assessment) (Figure 3).

Nematode community structure differed between soils of varying organic carbon content, pH and texture by both methods of assessment (Figure 2). However, there were differences between the methods as to which soil parameters were correlated with changes in nematode community structure. The T-RFLP method showed differences between soils of different organic carbon content and pH, though no differences in community were seen in soils of varying texture. However, the morphological assessment did show differences in the structure of nematode communities in soils of differing texture, with medium textured soils separating out from the other sites.

For nematode feeding types identified morphologically, the RMULTIVARIATE model accounted for 31% of the variance for plant feeding nematodes, 31% for omnivores, 44% for bacteriovores and 11% for predators. For fungal feeders the residual variance was greater than the variance of the response variate. Both RMULTIVARIATE and MLMM analyses revealed significant effects of biogeographical zone, texture and soil organic carbon on nematode community structure (Table 2). The only significant interaction was between soil organic carbon, texture and pH. For nematode feeding types identified from T-RFLP, the model accounted for 2% of the variance for plant feeding nematodes, 37% for omnivores, 30% for bacteriovores and 11% for fungal feeders, with no predators being identified. Biogeographical zone was identified as significantly affecting nematode community structure (Table 3), with an interaction between biogeographical zone and soil texture being identified by MLMM and between texture, pH and soil organic carbon by RMULTIVARIATE. Total nematode abundance showed significant interactions between land use and soil texture. Thus, land use (forestry) / texture (organic) P =

204 0.037; land use (grassland) / texture (medium) P = 0.011 and land use (grassland) / texture
205 (medium fine/fine/very fine) P = 0.007.
206
207 **4 Discussion**
208 The European sampling campaign was designed to allow an assessment of nematode community
209 structure as an indicator for soil monitoring through an investigation of the range of nematode
210 community structure types across different biogeographic region, under different land uses and
211 in soils of different characteristics.
212 The differences seen in nematode community structure across Europe strongly support the use of
213 bio-geographical zones to rationalise samples within a monitoring scheme at this scale. The
214 indicator schemes identified in the introduction (Faber et al., 2013, Griffiths et al., 2012) were
215 based at the national level, and are fit for purpose at that scale. However, Europe can be sub-
216 divided in many ways into different zones, based on climate, vegetation, and other factors
217 (Römbke and Breure, 2005). This makes it important when monitoring at the trans-national or
218 European level to take differences between bio-geographical zones into account, as normal
219 ranges of diversity in nematode community structure may create differences between zones.
220 These differences may mask changes in indicator values in response to stresses or perturbations
221 without proper calibration. Detailed analysis of nematode communities across different
222 European grassland types (Ekschmitt et al., 2001) suggests that discriminating different land-
223 uses as a factor within bio-geographical zones produces a greater level of accuracy for
224 monitoring nematode community structure. Our analysis, at the trophic group level, actually
225 showed no interactions between bio-geographical zone and land use. Thus the effects of land use
226 were consistent across the five bio-geographical zones studied. The only significant interaction,

from either the morphological or T-RFLP data, was between soil organic carbon, texture and pH. Given that texture and pH had no significant effects on their own, we interpret this as indicating the effects of soil organic carbon on nematode community structure are modified by texture and pH.

The methods used in this study are designed for the rapid screening of a large number of samples and are therefore rather coarse tools with which to investigate the structure of the nematode communities present. Variation in species diversity between sites is not explicitly captured by either method, both of which operate at a higher taxonomic level consistent with feeding group. Thus there may well be differences in species composition that are not detected at the feeding group level. In spite of this, the trends of clustering seen in the PCA data within bio-geographical zone, land use and soil characteristics indicate where the differences in nematode community structure within these groupings occur. These rapid screening methods therefore allow a large number of samples to be quickly investigated, and indicate which samples should be selected for intensive analysis at a later time with a method capturing species diversity (Griffiths et al., 2012) (either morphological identification to species, or species identification by sequencing of nematode DNA) or a method of assessment at family/order taxonomic levels as opposed to feeding groups.

The directed-T-RFLP method used (Donn et al., 2012) was originally developed to assess UK soil nematode assemblages. Though it has been shown to work in this instance at the European scale, additional data from other European nematodes would increase the taxonomic resolution. This would allow the examination of nematode community structure at the family or genera level, whilst retaining the rapidity and large volume of samples intrinsic to the method. The additional level of taxonomic resolution would be a major factor in the applicability of this

method to assess nematode community structure as an indicator for soil monitoring. A study by Wiesel et al. (2015) showed that the size of the soil sample used for the initial nematode extraction prior to T-RFLP needs to be in the order of 100g to ensure a representative sample. The time and expertise needed to carry out the morphological identification of samples to high taxonomic resolution is a constraint in many cases. Improvements in the application and throughput of molecular techniques would allow for larger scale monitoring (Thompson & Newmaster, 2014).

5 Conclusion

Nematode community structure varies between European bio-geographical zones, land-use and soil organic carbon categories., This should be taken into account when planning any trans-national or European soil monitoring scheme using nematodes as an indicator. The rapid morphological and molecular methods tested are an acceptable proxy screening for nematode community structure as they are sensitive to bio-geographical zone, land use and some soil characteristics. The rapid molecular directed- T-RFLP method has a greater ability to handle the large sample volumes needed for rapid sample screening. A more in-depth follow-up method is required for both methods, to provide the greater taxonomic resolution needed to compare species diversity and functional attributes.

Acknowledgements

This work was supported by the European Union within the project EcoFINDERS (FP7-264465). The James Hutton Institute and SRUC were partly funded by the Rural and

Environment Science and Analytical Services Division of the Scottish Government. The authors thank the anonymous referees for comments.

References

Breure, A.M., 2004, Soil biodiversity: measurements, indicators, threats and soil functions, International Conference: soil and compost eco-biology, September 15th – 17th 2004, León – Spain

Chen, X.Y., Daniell, T.J., Neilson, R., O'Flaherty, V., Griffiths, B.S., 2010. A comparison of molecular methods for monitoring soil nematodes and their use as biological indicators. European Journal of Soil Biology. 46, 319-324

Doncaster C.C. (1962). A counting dish for nematodes. Nematologica 7, 334–336.

Donn, S., Neilson, R., Griffiths, B.S., Daniell, T.J., 2011. Greater coverage of the phylum Nematoda in SSU rDNA studies. Biology and Fertility of Soils. 47, 333–339.

Donn, S., Neilson, R., Griffiths, B.S., & Daniell, T.J., 2012. A novel molecular approach for rapid assessment of soil nematode assemblages – variation, validation and potential applications. Methods in Ecology and Evolution. 3, 12-23

Faber, J.H., Creamer, R.E., Mulder, C., Römbke, J., Rutgers, M., Sousa, J.S., Stone, D., Griffiths, B.S., 2013. The Practicalities and Pitfalls of Establishing a Policy-Relevant and Cost-

295 Effective Soil Biological Monitoring Scheme, Integrated Environmental Assessment and
296 Management. 9:2, 276–284
297
298 Floyd, R.M., Rogers, A.D., Lambshead, P.J.D., Smith, C.R., 2005. Nematode-specific PCR
299 primers for the 18S small subunit rRNA gene. Molecular Ecology Notes. 5, 611–612.
300
301 Griffiths, B.S., Daniell, T.J., Donn, S., Neilson, R., 2012. Bioindication potential of using
302 molecular characterisation of the nematode community: Response to soil tillage. European
303 Journal of Soil Biology. 49, 92-97
304
305 ISO 23611-4:2007(E) Soil quality - Sampling of soil invertebrates - Part 4: Sampling, extraction
306 and identification of soil-inhabiting nematodes. International Organization for Standardization,
307 Geneva, Switzerland.
308
309 Massey, P., O'Connor, C., Sills, P., Fenelon, A., Maloney-Finn, L., Stone, D., Reidy, B. and
310 Creamer, R.E., 2014. Irish Soil Information System: Laboratory Standard Operating Procedures.
311 Technical Report (2007-S-CD-1-S1), published online by Environmental Protection Agency,
312 Ireland.
313
314 OECD, 2001, OECD expert meeting on Agri-Biodiversity indicators: Summary and
315 recommendations, 5-8 November 2001, Zurich, Switzerland.
316

Porazinska, D.L., Giblin-Davis, R.M., Powers, T.O., Thomas, W.K., 2012. Nematode Spatial and Ecological Patterns from Tropical and Temperate Rainforests. PLoS ONE. 7(9), e44641. doi:10.1371/journal.pone.0044641

Ritz, K., Black, H.I.J., Campbell, C.D., Harris, J.A., Wood, C., 2009. Selecting biological indicators for monitoring soils: A framework for balancing scientific and technical opinion to assist policy development. Ecological Indicators. 9, 1212-1221

Ritz, K., and Trudgill, D.L., 1999. Utility of nematode community analysis as an integrated measure of the functional state of soils: perspectives and challenges. Plant and Soil. 212, 1–11

Römbke, J., Breure, A.M., 2005. Ecological soil quality - Classification and assessment. Ecotoxicology and Environmental Safety. 62, 185-308

Stone, D., Blomkvist, P., Bohse Hendriksen, N., Bonkowski, M., Bracht Jørgensen, H., Carvalho, F., Dunbar, M.B., Gardi, C., Geisen, S., Griffiths, R., Hug, A.S., Jensen, J., Mendes, S., Plassart, P., Römbke, J., Rutgers, M., Schmelz, R. M., Sousa, J.P., Suhadolc, M., Winding, A., Creamer, R.E. 2015 (this issue). Establishing a Transect for Biodiversity and Ecosystem Function Monitoring Across Europe. Applied Soil Ecology. (in press).

Stone, D., Ritz, K., Griffiths, B.S., Creamer, R.E., 2015 (this issue). Selecting biological indicators appropriate for European soil monitoring. Applied Soil Ecology. (in press).

Thompson, K.A., Newmaster, S.G., 2014. Molecular taxonomic tools provide more accurate estimates of species richness at less cost than traditional morphology-based taxonomic practices in a vegetation survey. Biodiversity and conservation. 23, 1411–1424.

Turbé, A., De Toni, A., Benito, P., Lavelle, P., Ruiz, N., Van der Putten, W., Labouze, E. & Mudgal, S., 2010. Soil biodiversity: functions, threats, and tools for policy makers. BioIntelligence Service, IRD, and NIOO, Report for European Commission (DG Environment), Brussels, Belgium. 250 p

Wiesel, L., Daniell, T.J., King, D., Neilson, R., 2015. Determination of the optimal soil sample size to accurately characterise nematode communities in soil. Soil Biology & Biochemistry. 80, 89-91.

Yang, C.X., Ji, Y.Q., Wang, X.Y., Yang, C.Y., & Yu, D.W., 2013. Testing three pipelines for 18S rDNA-based metabarcoding of soil faunal diversity. Science China Life Sciences. 56(1), 73-81.

Yoder, M.; Tandingan De Ley, I., King, I.W., Mundo-Ocampo, M., Mann, J., Blaxter, M., Poiras, L., and De Ley, P., 2006. DESS: a versatile solution for preserving morphology and extractable DNA of nematodes. Nematology. 8(3), 367-376

361 Table 1. Nematode abundance (no g^{-1} dry soil with standard deviation) of the 81 sites sampled,
362 by biogeographical zone, land use and soil parameter. *Organic Carbon Content was not
363 determined for site POR_01 as there was not enough sample to allow this test.

Category	Number of sites	Nematode Abundance (per gram dry soil)	Std Dev
Bio-geographical Zone			
Alpine	12	25.8	8.1
Atlantic	33	24.1	18.5
Boreal	4	27.7	9.4
Continental	27	23.0	15.9
Mediterranean	5	8.3	6.7
Landuse			
Arable	27	13.8	15.2
Forestry	19	25.6	13.5
Grassland	35	29.2	14.2
Soil pH			
Acidic (<pH 5)	12	27.7	8.3
Neutral (pH 5-7)	41	23.3	16.9
Alkaline (>pH 7)	28	20.0	15.8
Soil Organic Carbon Content *			
< 2 % (Mineral Soils)	22	12.5	6.7
2 % - 15 % (Organo-mineral soils)	51	26.6	16.9
> 15 % (Organic soils)	7	31.3	12.1
Soil Texture			
Coarse	15	24.3	20.6
Medium	26	23.1	16.4
Medium Fine /Fine/Very Fine	32	21.0	13.2
Organic	7	31.2	12.2

364

Table 2. Degrees (df) and numerated degrees of freedom (n.df), and the significance value (P) from regression analysis using a multivariate analysis of variance approach for unbalanced designs (RMULTIVARIATE) and multivariate linear mixed models (MLMM) of nematode feeding groups identified morphologically against the categories outlined in Table 1 (biogeographical zone (Zone), land use, soil texture, soil pH and soil organic carbon content (orgC)). Only combinations of categories that had a significant bearing on the regression outcome are presented, combinations with no significant bearing are not presented or labelled as n/a (not applicable).

Category	**RMULTIVARIATE**		**MLMM**	
	df	**P**	**n.df**	**P**
All terms	45	0.009	33	<0.001
Zone	4	0.013	20	0.008
Land Use	2	0.021	10	0.016
Texture	3	0.438	15	0.372
pH	2	0.339	10	0.285
orgC	1	0.000	5	<0.001
Zone/Land Use	6	0.160	30	0.109
Zone/Texture	7	0.644	n/a	n/a
Zone/pH	3	0.142	20	0.242
Zone/orgC	n/a	n/a	20	0.827
Land Use/Texture	5	0.379	25	0.372
Land Use/pH	3	0.089	15	0.142
Texture/pH	4	0.252	20	0.172
Texture/orgC	2	0.943	10	0.889
pH/orgC	1	0.439	5	0.382
Zone/Land Use/Texture	1	0.050	n/a	n/a
Zone/Land Use/pH	n/a	n/a	5	0.11
Texture/pH/orgC	1	0.025	5	<0.001

Table 3. Degrees (df) and numerated degrees of freedom (n.df), and the significance value (P) from regression analysis using a multivariate analysis of variance approach for unbalanced designs (RMULTIVARIATE) and multivariate linear mixed models (MLMM) of nematode feeding groups identified by directed-T-RFLP against the categories outlined in Table 1 (biogeographical zone (Zone), land use, soil texture, soil pH and soil organic carbon content (orgC)). Only combinations of categories that had a significant bearing on the regression outcome are presented, combinations with no significant bearing are not presented or labelled as n/a (not applicable).

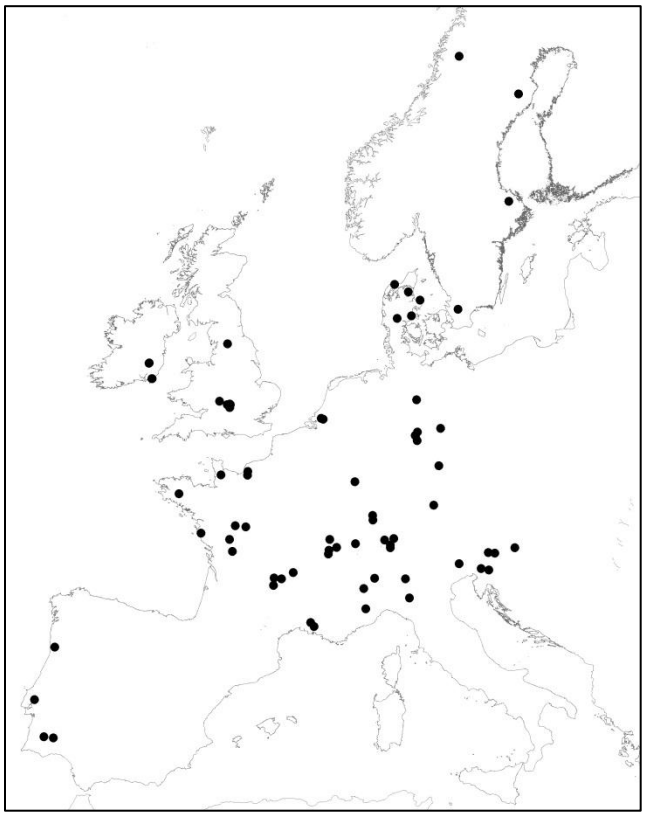
Category	**RMULTIVARIATE**		**MLMM**	
	df	**P**	**n.df**	**P**
All terms	46	0.010	54	<0.001
Zone	4	0.000	16	<0.001
Land Use	2	0.059	8	0.082
Texture	3	0.582	12	0.599
pH	2	0.184	n/a	n/a
orgC	1	0.260	n/a	n/a
Zone/Land Use	6	0.143	24	0.115
Zone/Texture	7	0.053	28	0.041
Zone/pH	3	0.549	n/a	n/a
Zone/orgC	1	0.157	n/a	n/a
Land Use/Texture	5	0.068	n/a	n/a
Land Use/pH	3	0.403	n/a	n/a
Land Use/orgC	1	0.191	n/a	n/a
Texture/pH	4	0.597	n/a	n/a
Texture/orgC	2	0.750	n/a	n/a
pH/orgC	1	0.643	n/a	n/a
Texture/pH/orgC	1	0.048	n/a	n/a

Figure 1. Distribution of the 81 sites sampled across Europe

Figure 2 PCA plots of nematode community structure differences based on nematode feeding groups (group means and SEM) identified by: a) nematode trophic group data from T-RFLP peaks, b) nematode trophic group data from morphological identification. i) within bio-geographical zones, ii) within land use categories, iii) within soil texture categories, iv) within soil pH categories, v) within soil organic carbon categories, vi) loadings

Figure 3. PCA plots of nematode community structure differences based on nematode feeding groups (group means and SEM) identified by: a) nematode trophic group data from T-RFLP peaks, b) nematode trophic group data from morphological identification. i) Bio-geographical zones within Arable land use, ii) Bio-geographical zones within Forestry land use, iii) Bio-geographical zones within Grassland land use.

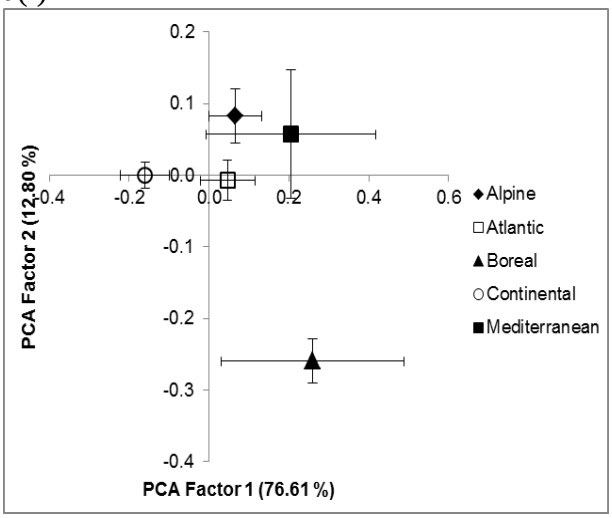
401 Figure 1.



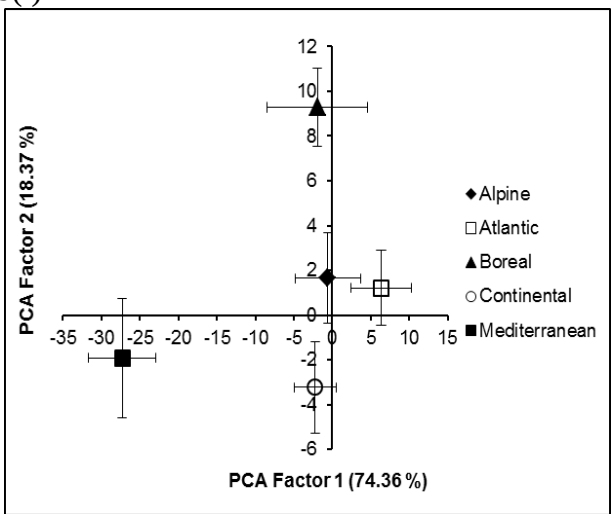
402
403

Figure 2

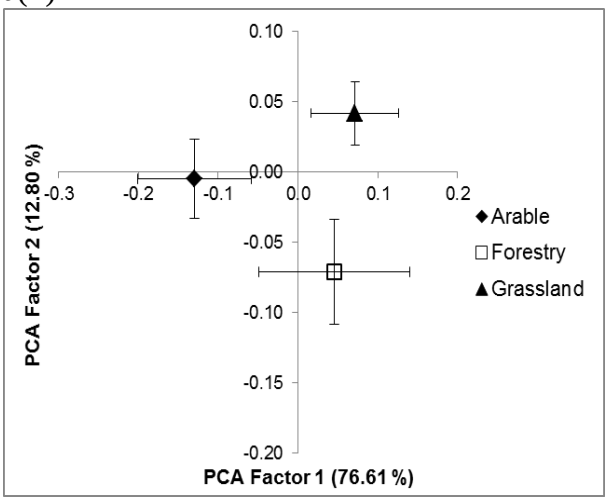
a(i)



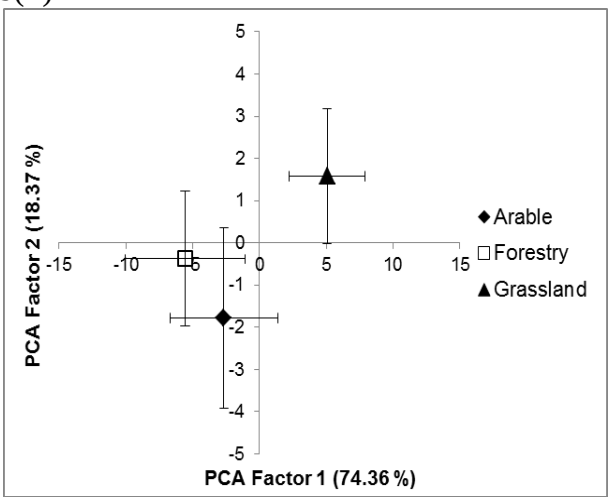
b(i)



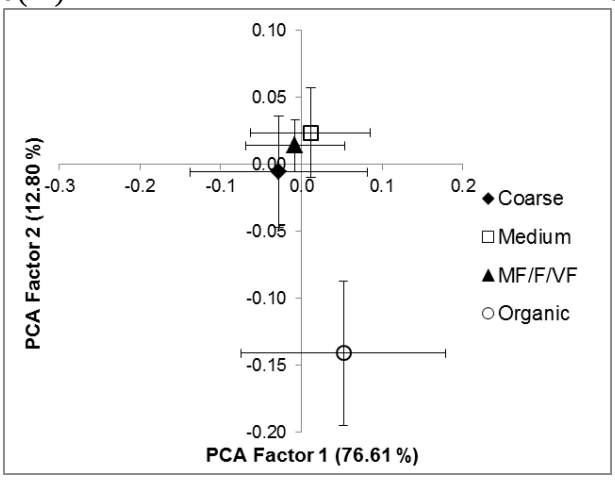
a(ii)



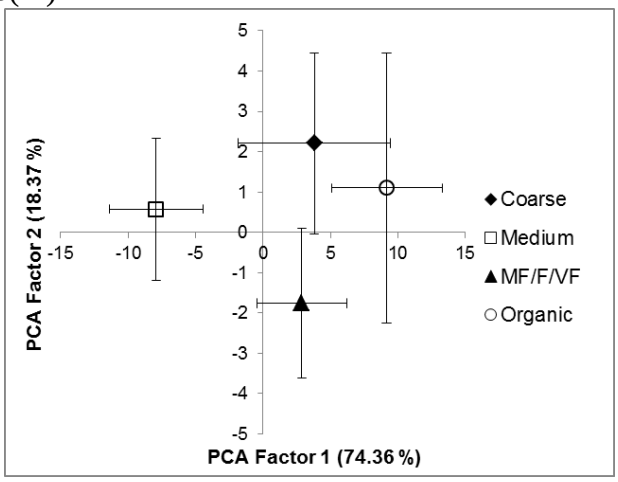
b(ii)



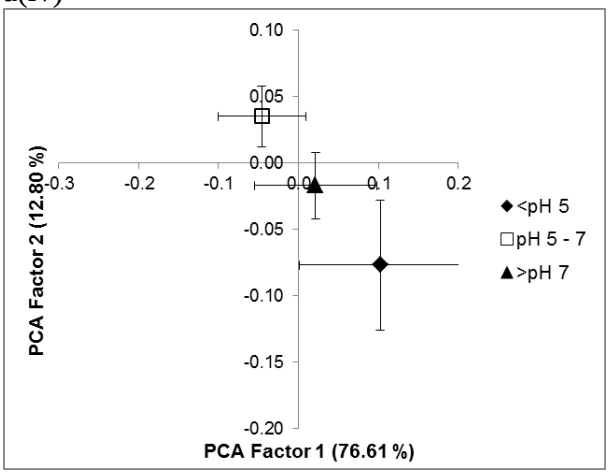
a(iii)



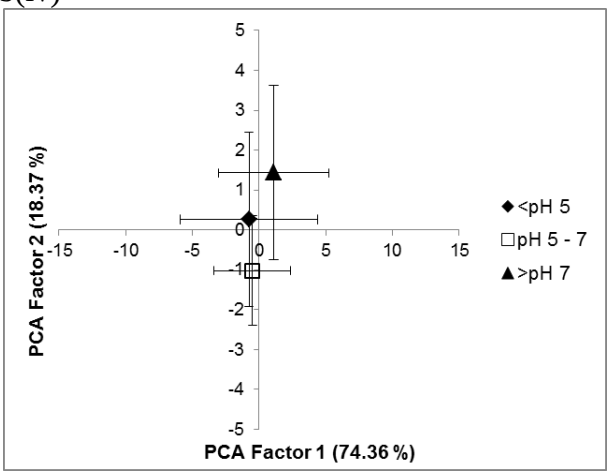
b(iii)



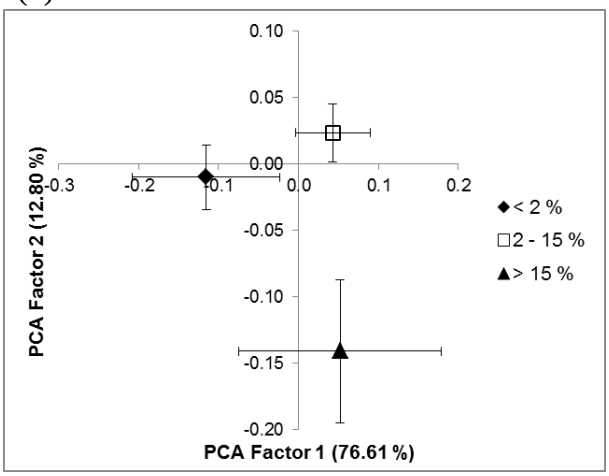
a(iv)



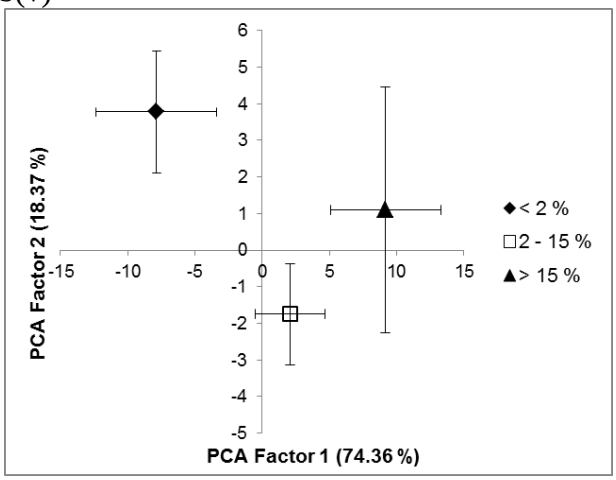
b(iv)



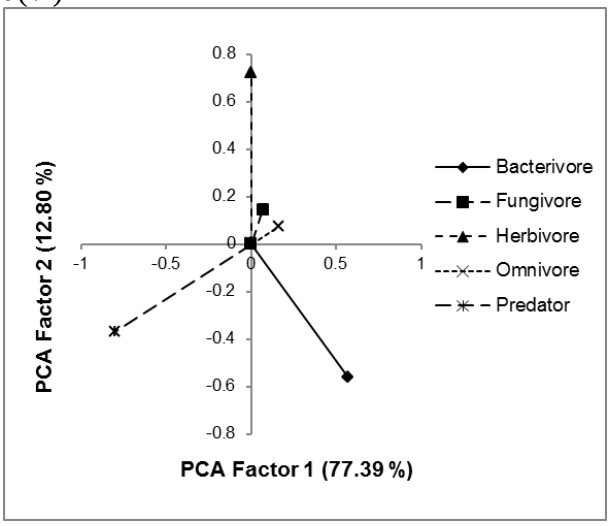
a(v)



b(v)



a(vi)



b(vi)

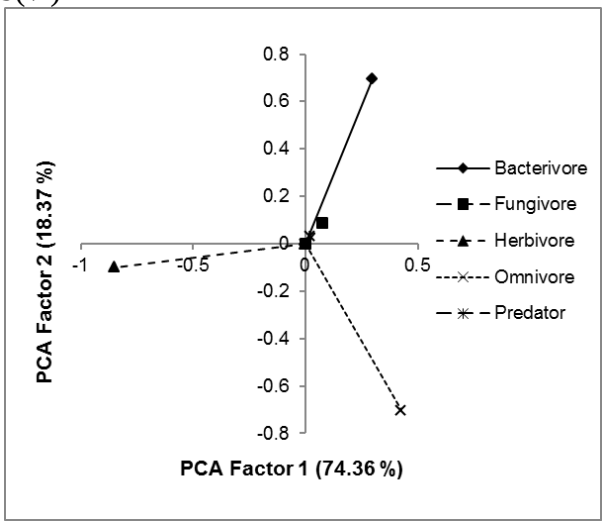
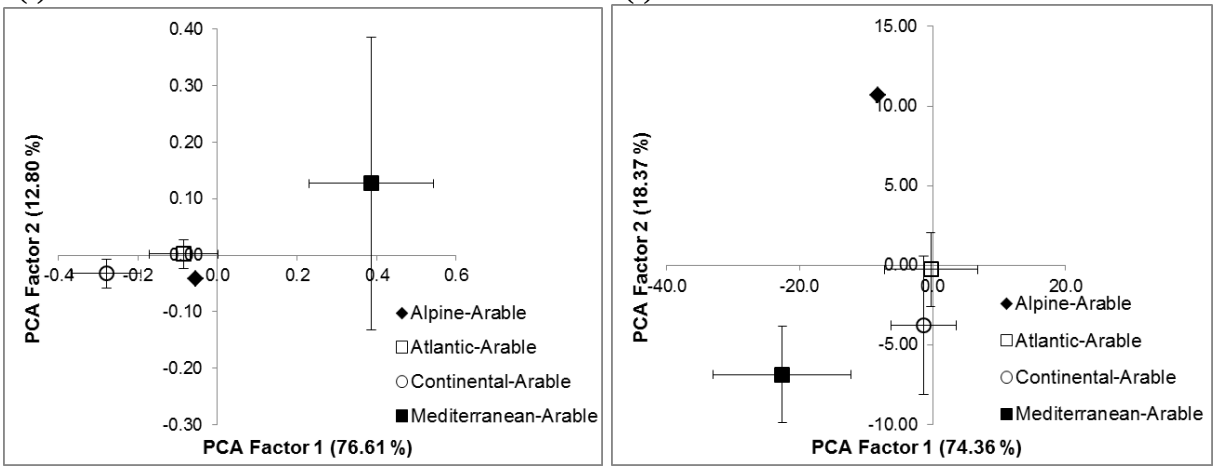
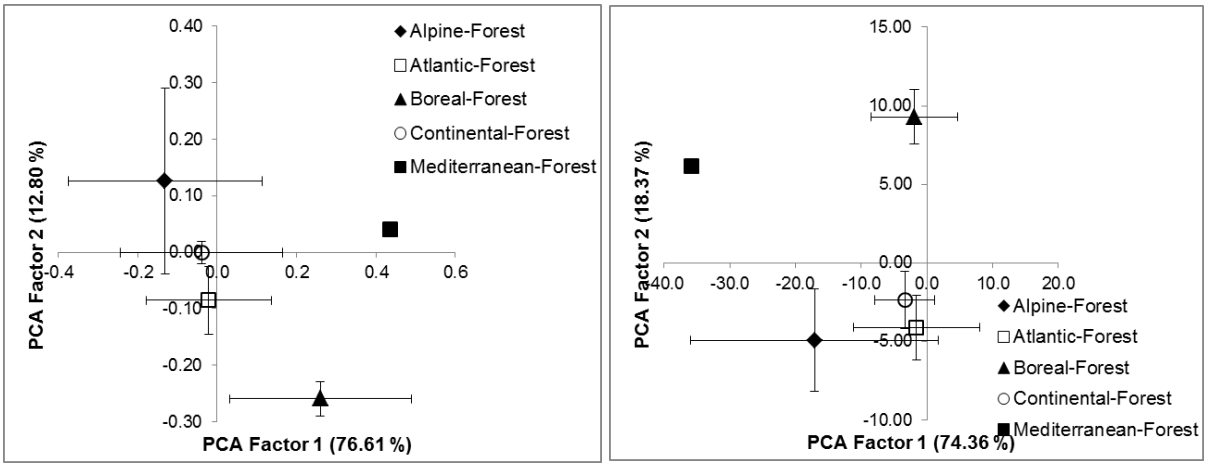


Figure 3.

a(i) b(i)



a(ii) b(ii)



a(ii) b(ii)